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ORIGINAL PAPER

Optimization of process parameters for the production of carbonyl reductase by *Candida viswanathii* in a laboratory-scale fermentor

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Abstract The effect of pH, aeration and mixing on the growth and production of carbonyl reductase by *Candida viswanathii* was investigated in a 6.6-l fermentor. Controlling the pH at 8.0 had a very significant effect on the enzyme production. Aeration and agitation influenced the dissolved oxygen concentration which in turn affected growth as well as enzyme production. A maximum carbonyl reductase activity (53 Umg^{-1}) was attained in 24 h under the optimal cultivation conditions of controlled pH at 8.0, aeration rate 1 vvm and an agitation speed of 250 rpm at 25°C. The enzyme activity was twice as high (56 Umg^{-1}) in the fermentor as compared to a shake flask. Further, the duration of growth and enzyme production in the fermentor was shortened. Cells cultivated under the optimized conditions were used for the preparative scale reduction of N, N-dimethyl-(3-keto)-2-thienyl-propanamine to (*S*)-N, N-dimethyl-(3-hydroxy)-2-thienyl-propanamine, a key intermediate in the production of the important antidepressant drug (*S*)-duloxetine.

Keywords Carbonyl reductase · *Candida viswanathii* · Fermentor · pH · Aeration and agitation rates

Introduction

Chiral hydroxyl compounds are the building blocks for the synthesis of many pharmaceuticals, inhibitors and pheromones of high-enantiomeric purity and are in great demand [12]. Today, these chiral alcohols are prepared on the laboratory scale by microbial reduction of the corresponding keto compounds. Chemical yields and the stereoselectivity of the processes are highly dependent upon the source and the physiological state of the microorganism used [10]. Historically, microbial reductions are predominantly carried out by baker's yeast [13, 23]. However, these processes are quite inefficient as in certain cases though the conversion of the substrate to product is good, the enantiomeric excesses are moderate to poor, the reason being the presence of more than four carbonyl reductases in the baker's yeast [25]. Recently, a number of other microorganisms have been reported which can carry out the stereoselective oxidation and reduction with good ee [3, 5, 9, 15]. We had isolated a potent carbonyl reductase producing yeast strain, *Candida viswanathii* MTCC 5158 using an enrichment and isolation procedure [19]. This yeast strain was used for the stereoselective reduction of a number of prochiral ketones to give the corresponding chiral alcohols with good yield and excellent (*S*)-enantioselectivity [6, 17, 18, 20, 21].

Little information is available regarding the production of carbonyl reductase in a bioreactor. Optimization of the fermentation conditions in the reactor is quite important for the large-scale production of carbonyl reductase, which in turn is required for the scale up of the bioreduction process. The rate and the amount of carbonyl reductase produced in a batch culture may vary under different physicochemical conditions. The choice of an appropriate pH, agitation and aeration level are crucial, due to the strong influence of these variables in the culture yield and operating cost.

It is well known that the pH, agitation and aeration rates affect the productivity of the fermentation process [8, 11, 14]. The effect of agitation on morphology, cell growth and product yield has been investigated for several fermentation processes. Agitation speed plays an important role in the

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improvement of mass and heat transfer in the fermentor [16]. An increase in the agitation speed improves mixing, mass and heat transfer, but may also have negative effects such as disrupting of the cells, vacuolation and autolysis, which in turn decrease the overall productivity [1]. Similarly, pH and aeration rates are also important parameters for the process scale up [4, 7].

The present study is aimed at investigating the desired combination of pH, aeration and agitation that would yield the highest growth and carbonyl reductase activity by *C. viswanathii*. The cells were grown under the optimized conditions and the resting cells were utilized to carry out the preparative scale reduction of N, N-dimethyl-3-keto-(2-thienyl)-propanamine (DKTP) hydrochloride to produce (*S*)-N, N-dimethyl -3-hydroxy-(2-thienyl)-propanamine (DHTP), a key intermediate of (*S*)-duloxetine.

Materials and methods

The yeast strain isolated from brewery waste by an enrichment technique and identified as *C. viswanathii* was used in the present study [12]. The growth and maintenance of the organism were carried out using the standardized procedure as described previously [22]. The production medium (pH 9.0) for the fermentation studies was prepared by supplementing the nutrient broth with 1% (wv^{-1}) mannitol, 0.5% (wv^{-1}) yeast extract, 4 mM Ca^{2+} and 2 mM acetophenone. For inoculum preparation, two 500-ml shake flasks, each containing 100 ml production medium was inoculated with 3 ml freshly prepared *C. viswanathii* culture. The culture was incubated for 18 h at 25°C in an incubator shaker (200 rpm) and was used to inoculate the fermentor.

Production studies in laboratory-scale fermentor

A 6.6-l fermentor (BioFlo 300, New Brunswick Scientific Co., USA) containing 4.5 l production medium was used to study the effect of pH, aeration and agitation. The fermentor was inoculated with 10% (vv^{-1}) inoculum. Agitation was provided with six-bladed Rushton impeller (impeller diameter was one-third of the vessel diameter). Four side-walled equidistant baffle plates were used to prevent vortex formation. The pH was monitored using a glass electrode immersed in the fermentation broth. Dissolved oxygen was measured using a polarographic electrode (Ingold, Leicester, UK). Calibration (the % of atmospheric oxygen) was performed with air-saturated medium (100%) and nitrogen-saturated medium (0%) after sterilization. Polypropylene glycol was added when foaming occurred. Batch experiments were carried out at 25°C under uncontrolled and controlled pH, at varying aeration and agitation rates to optimize the process parameters with the ultimate aim to maximize the growth and carbonyl reductase production. Experiments were conducted at uncontrolled and controlled pH (7, 8, 9 and 10). The agitation rate was adjusted to 150, 250 and 350 rpm, while the aeration rate was varied from 0.5 to 1 vvm. Samples from the fermentor were withdrawn at regular intervals and analyzed for growth, total protein content and carbonyl reductase activity.

Preparative scale bioreduction of DKTP

For the preparative scale bioreduction of DKTP, the biomass obtained after 24-h fermentation under controlled optimal condition was harvested and suspended in sodium phosphate buffer (0.2 M, pH 7). To the resting cell suspension (250 g/l), 1 g DKTP hydrochloride solution in water was added. The reduction was completed after 60 h at 30°C (200 rpm). The enantiopure DHTP was obtained from the reaction mixture by solvent extraction and silica gel chromatography as described previously [17].

Analysis

For the estimation of growth, the samples were centrifuged at 7,000×*g* for 10 min, and the precipitated cell mass was washed with distilled water. Centrifugation and washing were repeated twice. The mass of the wet cell pellet was determined and was expressed in grams per liter of culture broth. The carbonyl reductase activity was determined spectrophotometrically by monitoring the decrease in absorbance of NADH at 340 nm [24] using acetophenone as a model compound. The standard reaction mixture (1 ml) contained 0.1 mM NADH, 50 µl cell free extract and 2.5 mM ketone substrate in dimethyl sulfoxide. The cell-free extract was prepared after lysis of the cell suspension (166 mg ml^{-1}) in phosphate buffer (50 mM, pH 7) by ultrasonication for 15 min (a pulse of 30 s followed by rest of 30 s). Cell debris was removed by ultracentrifugation at 50,000×*g* for 30 min at 4°C. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol NADH at 25°C. Total protein concentration in all samples was estimated by Bradford's dye binding assay [2] using bovine serum albumin (1 mg ml^{-1}) as the standard. All the assays were carried out in triplicate along with appropriate buffer and reagent control. The absorbance was monitored at 595 nm. All the biocatalytic reaction profiles were analyzed by HPLC (Shimadzu, Japan). Aliquots of the reaction mixture were centrifuged (10,000×*g*, 5 min) to separate out the cells. The supernatant was extracted twice using ethyl acetate as the solvent. The organic solvent was dried and removed under reduced pressure and subjected to HPLC analysis according to the reported method [17].

Results and discussion

Production studies without pH control

Culture pH is one of the most important process parameters that have been reported to affect microbial growth and enzyme yield. In order to determine how the pH affects the growth and carbonyl reductase production by *C. viswanathii*, various fermentor runs were conducted at a varying initial pH, 7, 8, 9 and 10, at 25 °C with aeration and agitation rates of 0.75 vvm and 200 rpm, respectively, in a 6.6-l fermentor (working volume 4.5 l). The initial pH of the medium was adjusted to the required value, after which it was monitored but not controlled. During these studies it was observed (Fig. 1) that as growth progressed, the pH of the medium rapidly decreased by 1–2 U within 6–12 h irrespective of initial pH, after which it stabilized for the rest of the fermentation run. Maximum carbonyl reductase yield (36.12 Umg^{-1}) was obtained when the initial pH of medium was kept at 9. As the initial pH of the medium was adjusted toward lower (pH 7) or higher (pH 10) pH values, there was a considerable decrease in enzyme production, which was proportional to the growth. During these studies, a significant increase in both growth as well as enzyme production was observed as compared with that in the shake flasks. These studies suggested that an initial pH of 9.0 was a prerequisite for the culture growth and as the growth progressed, a decline in pH correlated with the subsequent increase in the carbonyl reductase production. Therefore, to further confirm this aspect, various fermentor runs were carried out under controlled pH condition.

Production studies with pH control

To obtain a better understanding of the effect of pH on growth and carbonyl reductase production by *C. viswanathii*, various fermentor runs were taken under controlled pH environment (7, 8, 9 and 10) at 25°C with aeration and agitation of 1 vvm and 250 rpm, respectively. After the inoculation of the fermentor, throughout the run, the pH of the fermentation broth was controlled at its initial pH level using 2 N NaOH. As observed from the production data (Fig. 2), it is clear that the maximum growth (34 g/l at 24 h) as well as carbonyl reductase production (54 Umg^{-1} at 24 h) was achieved when the fermentation was carried out at a controlled pH 8.0. The growth and enzyme production at controlled pH 7 was 30 gl^{-1} and 26 Umg^{-1}, respectively, after 24 h. Both growth and enzyme production at controlled pH 9 and 10, were significantly lower as compared to pH 8. These data suggest that the highest growth as well as enzyme production achieved at uncontrolled pH 9 might be due to the decline in pH from the initial value (pH 9) by

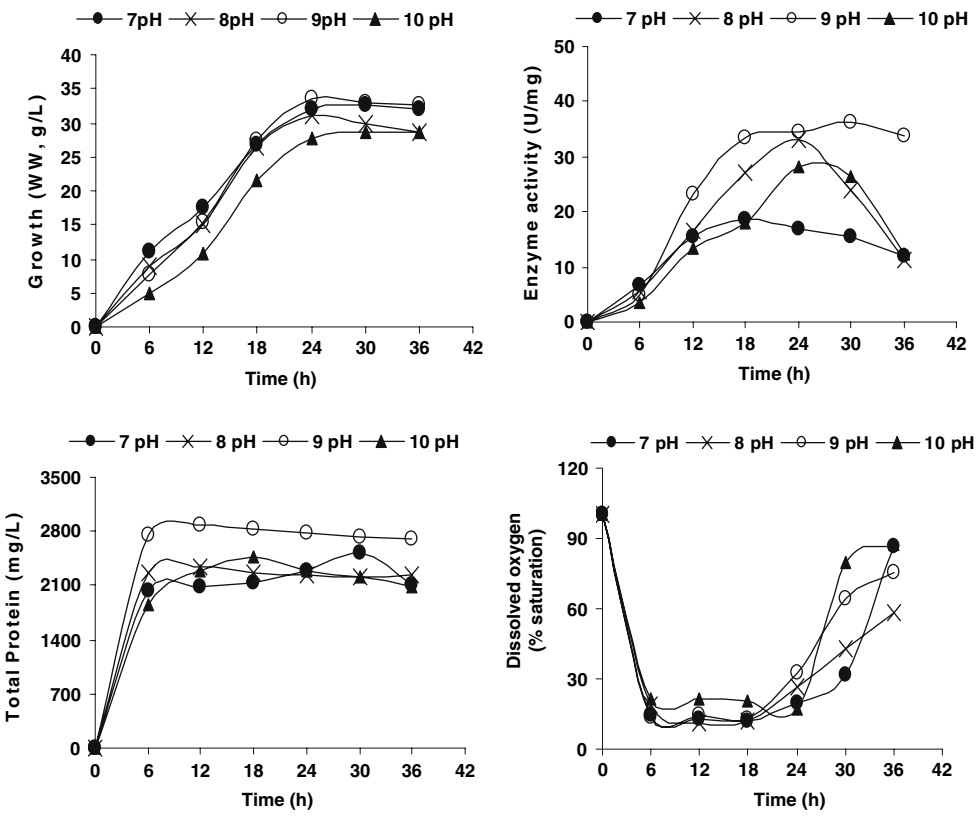

Fig. 1 Time course of growth, DO, total protein and enzyme production by *C. viswanathii* in a stirred tank fermentor without pH control

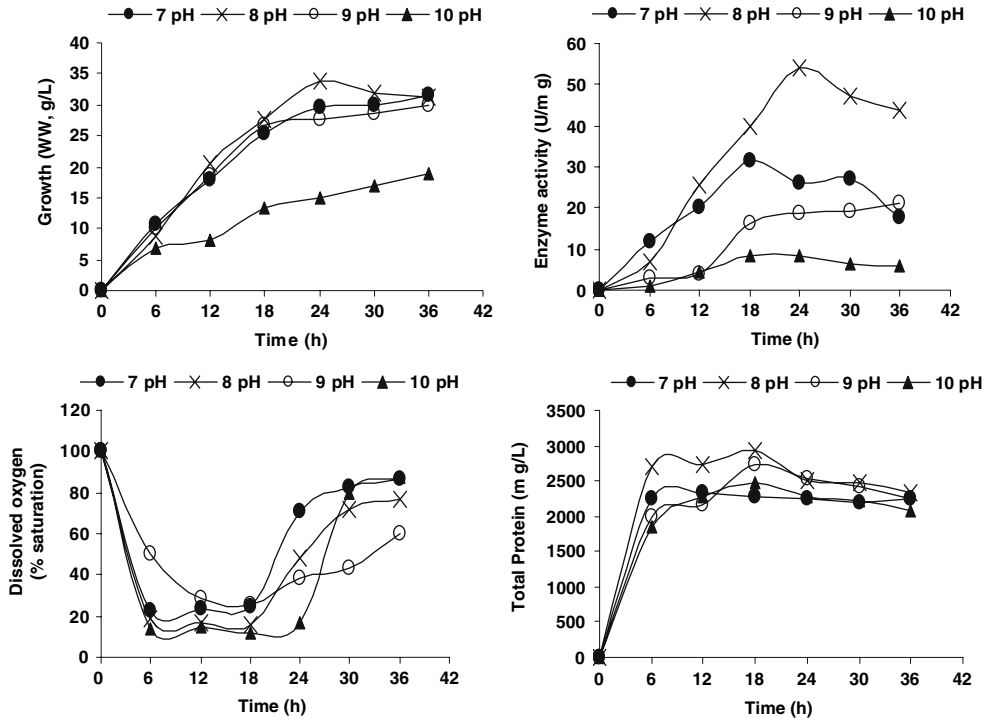

Fig. 2 Time course of growth, DO, total protein and enzyme production by *C. viswanathii* in a stirred tank fermentor with pH control

about 1–2 U within the first 6 h of fermentation. This showed that the maximum growth as well as enzyme production by *C. viswanathii* occurred when the pH of the production medium was in the range of 7–8.

Effect of agitation

The mass transfer and gas dispersion play a critical role in a successful fermentation. The agitation rate and hence the shearing action of impellers has significant effect on growth as well as on enzyme production in a fermentor. To study the effect of shearing rate on the growth and carbonyl reductase production by *C. viswanathii*, batch fermentations were carried out at different agitation rates (150, 250 and 350 rpm) at a controlled pH with 1 vvm aeration (25°C). It is evident from Fig. 3 that there is a considerable increase in both growth and enzyme production with an increase in agitation rate from 150 to 250 rpm. The maximum carbonyl reductase activity achieved was 54 Umg^{-1} at 250 rpm. It is interesting to note that the carbonyl reductase production showed its growth-associated nature. There is a good chance that the process with high growth may ensure a high carbonyl reductase production. Also, both the growth and carbonyl reductase production are the functions of mixing rate. However, when the agitation rate was increased beyond 250 rpm, a significant decrease in growth was observed. This might be due to the higher shear stress caused by the blade tips of the impeller, which increases with agitation rate. Additionally this effect is pernicious for the carbonyl reductase production, since for agitation rates greater than 250 rpm, there is an effective decrease of the enzyme production. A high agitation speed might have increased the dispersion of the macromolecules in the medium, thus leading to an increased growth and enzyme production. However, shearing effect induced by the higher agitation speed on the cells may contribute negatively toward cell growth and enzyme production. K_La was determined using the dynamic gassing out method at different agitation rates. The aeration capacity of the fermentor was highest at 350 rpm with a K_La of 56 h^{-1} and a significant decline in K_La was observed with the decrease in agitation rate. The values of K_La at 150 and 250 rpm were determined to be 25 and 45 h^{-1}, respectively.

Effect of aeration

The effect of aeration was investigated at three aeration rates, namely 0.5, 0.75 and 1 vvm, by maintaining the operating pH and agitation speed at 8.0 and 250 rpm, respectively (Fig. 4). The maximum enzyme production at the aeration rates of 0.5, 0.75 and 1 vvm, were 9, 50 and 56 Umg^{-1}, respectively. Comparing the growth and enzyme production at different aeration rates, the growth-associated nature of enzyme production could still be observed. The dissolved oxygen concentration profiles under different aeration rates were significantly different. At a lower aeration rate of 0.5 vvm, the dissolved oxygen

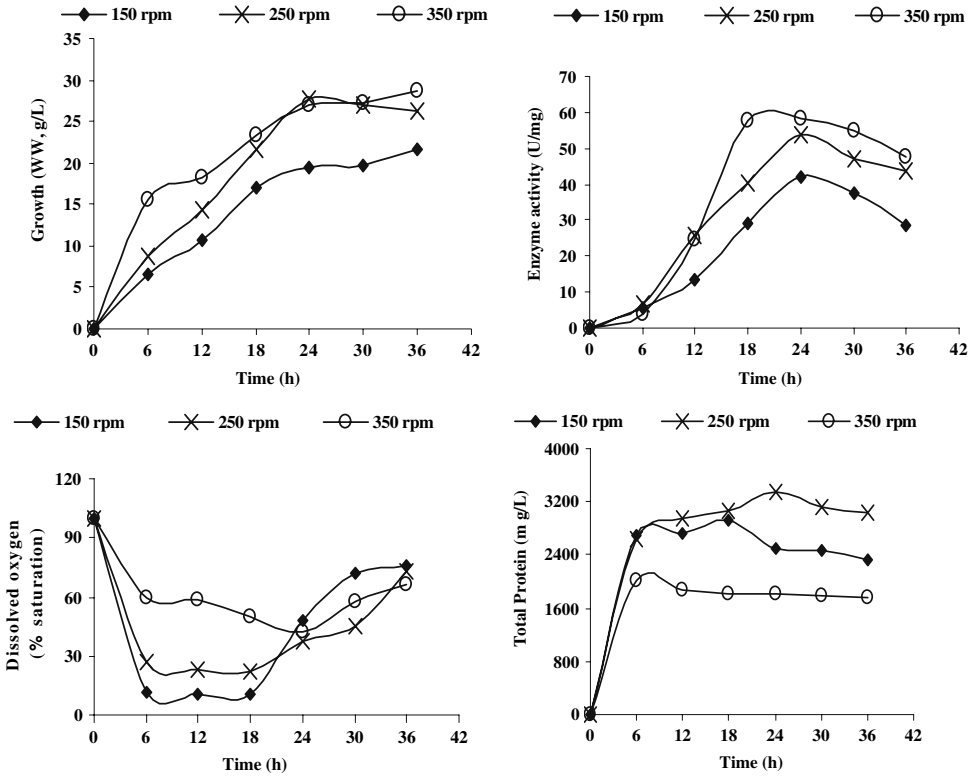

Fig. 3 Effect of agitation on the growth and carbonyl reductase production by *C. viswanathii*

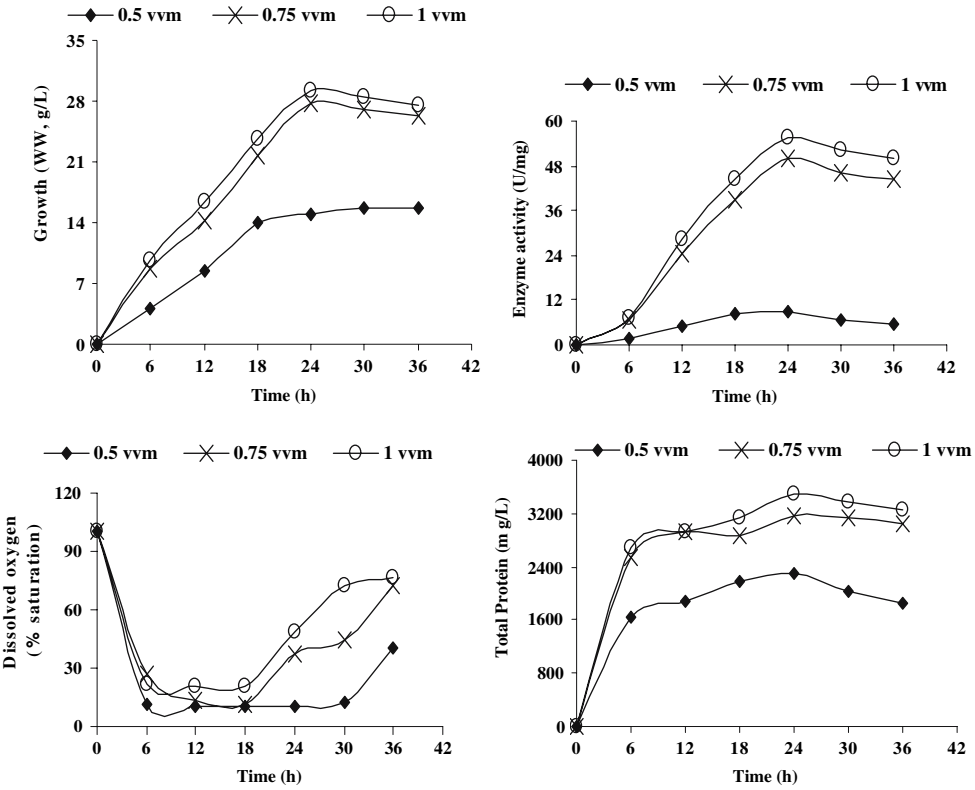

Fig. 4 Effect of aeration rate on the growth and carbonyl reductase production by *C. viswanathii*

concentration was less than 10% saturation for most of the time. The dissolved oxygen concentration in case of 1 vvm aeration was above 30% throughout the fermentation run. Oxygen limitation was probably the reason for the lower cell mass and enzyme production at the lower aeration rates. These results indicate that aeration could influence dissolved oxygen concentration significantly, which in turn would affect cell growth and carbonyl reductase production.

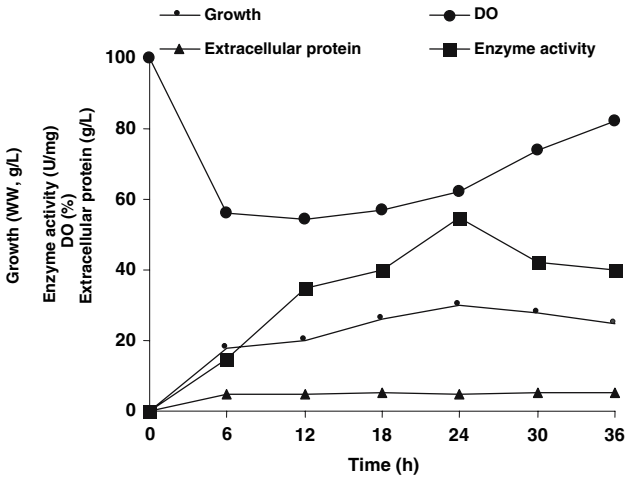

Fig. 5 Time course of growth, DO, total protein and carbonyl reductase production by *C. viswanathii* in a stirred tank reactor under optimal condition

Optimal carbonyl reductase production by *C. viswanathii*

A typical batch cultivation time course for the growth and enzyme production in a 6.6-l fermentor is shown in Fig. 5. Growth reached a maximum value of 29 gl^{-1} at 24 h. Extracellular protein production and carbonyl reductase activity were associated with the microbial growth and reached a maximum level (3.5 gl^{-1} and 56 Umg^{-1}, respectively) at 24 h. The production process in shake flask was compared with that of 6.6-l fermentor. Maximum growth (29 gl^{-1}) and carbonyl reductase activity (56 Umg^{-1}) in the fermentor were 168 and 205% higher, respectively, than those obtained in shake flask (data not shown). Furthermore, the productivity obtained in the 6.6-l fermentor (5,612 $Ul^{-1}h^{-1}$) was 218% higher than that in the shake flask (data not shown).

Preparative scale bioreduction of DKTP

Gram scale reduction of DKTP with the fermentor-grown cells was carried out under the optimized reaction conditions as described previously [17]. To a resting cell suspension (250 gl^{-1}) in sodium phosphate buffer (0.2 M pH 7.0), 1 g DKTP as a hydrochloride salt dissolved in distilled water was added. The reaction mixture was incubated at 30°C for 60 h (200 rpm). Under these conditions, (*S*)-DHTP at a conversion of about >81 and >99% ee was obtained.

Conclusion

A remarkable improvement in microbial growth as well as enzyme production is obtained in the stirred tank bioreactor compared to the shake flasks. Besides, no operational problems are detected suggesting the possibility of applying this process to large-scale system for the production of (*S*)-DHTP, a key intermediate of the chiral antidepressant drug, (*S*)-duloxetine (Table 1).

Table 1 Estimation of volumetric oxygen transfer coefficient (K_La) by dynamic gassing out method at varying agitation speed

Agitation (rpm)	K_La (h^{-1})
150	25
250	45
350	56

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